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RESEARCH ARTICLE

Generation of HSC-Like Cells from Human Embryonic Stem Cells by Inhibition of TGF-βR2 Signaling

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Abstract The efficient induction of embryonic stem cells into hematopoietic stem cells (HSCs) requires understanding the pro or inhibitory regulatory networks, which are involved in these pathways. Transforming growth factor β family is a super family of growth factors such as TGF-β1, TGF-β2, TGF-β3, and some other cytokines. Previous works indicate that some of TGF-β subfamilies have negative regulatory effect during hematopoietic differentiation and HSC proliferation. The inhibitory effects of TGF-β1 in early stage of differentiation of endothelial and hematopoietic cells have been shown. Therefore, in the present study, in order to evaluate the promotion of hematopoietic differentiation, the activities of TGF-β regulatory factor were reduced through knockdown of TGF-βR2 expression. The ESCs were transduced by *Lentivirus* carrying SiTGF-βR2 and its scramble separately. Green fluorescent protein (GFP) was used as a marker to monitor gene expression. Six days post transduction GFP^+ colonies were used for EB generation and the next day, the same size EBs were transferred in differentiation media involved in defining cytokines. The expression level of hematopoietic-specific genes was evaluated in 0, 7, and 15-days of differentiation. The real-time PCR analysis in 15 day EBs showed 2–3-folds increase in SiTGF-βR2 as compared with the scramble. Therefore, this finding may be confirmed if TGF receptor silencing can reduce the inhibitory effect of TGF-β during early hematopoietic differentiation.

Keywords ESCs · HSCs · TGF-βR2 signaling

Introduction

Today, transplantation of hematopoietic stem cells (HSCs) to develop multi lineage hematopoietic cell engraftment is the most common clinical cell-based therapy. Currently bone marrow, umbilical cord blood, and mobilized peripheral blood are the major sources of transplantable HSCs. The limited cell dose, the lack of additional immune cells if donor lymphocytes are needed and resultant delayed engraftment are some disadvantages of HSC transplantation [1]. To overcome these problems researchers need an appropriate alternative for generation of hematopoietic cells [2].

Embryonic stem cells (ESCs) are pluripotent cells established from the inner cell mass of blastocyst-stage embryos, in both mouse and human beings [3]. They can be differentiated into any of the cell types in three-germ layers [4, 5]. These characteristics make ESC attractive as the cell source for generation of hematopoietic cell. There are numerous reports on the production of HSCs or hematopoietic progenitors cells (HPCs) from ESCs or induced pluripotent stem cells (iPSCs). The human ES cell (hESC) differentiation system provides an outstanding alternative to the early embryo for identification of the hemangioblast as it allows easy access to large number of cells representing the earliest stages of hematopoietic commitment [6].

There are two major approaches in differentiation of ESC to HSC. First being non-genetic approach which is focused on mimicking the hematopoietic niche; second related to manipulation of the expression of genes

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responsible for hematopoietic development [7]. To achieve this goal different techniques have been applied, including the use of feeder layers [8], embryoid body (EB) formation [9], cytokine cocktails, and a combination of these techniques [8, 9]. Although it has been proved particularly difficult to produce the most potent HSCs capable of long-term repopulating HSCs (LTR-HSCs) that can reconstitute bone marrow and generate fully mature and functional cells [10]. So, identification of genes and key signaling pathways that regulate the hematopoietic development of ESC have been extensively studied over the past decade to determine optimal conditions for in vitro expansion of HSCs in the hope of increasing the number of engraftable LTR-HSCs.

The transforming growth factor β (TGF-β) family is a super-family of growth factors such as TGF-β1, TGF-β2, TGF-β3, and some other cytokines [11]. These members bind to receptor complexes composed by TGFβ receptor type I, activating receptor I, and receptors type II (TGFβRII, BMP-R II, and activin-RII), also called activin-like kinase receptor (Alk) [12]. It is well-known that TGF-β exerts multiple biological effects in regulating a wide variety of biological processes, namely: cell growth, apoptosis, differentiation, migration, extracellular matrix (ECM) production, immunity, angiogenesis, tumor metastasis, invasion, and embryonic development [13]. TGF-β also is a regulator of all stages of hematopoiesis [14] which shows pro- or anti-effect during differentiation stage. Previous works indicate that TGF-β1, one of subfamilies, has inhibitory effect in early stage of differentiation of endothelial and hematopoietic cells which are derived from a common precursor [12, 15]. Some reports demonstrated that TGF-β inhibition could maintain the proliferation and vascular identity of purified endothelial cells derived from hESCs. Further studies are needed to investigate how TGF-β regulates hematopoietic cell differentiation.

In the present study TGF-βR2, as one of the important receptor of TGF-β subunits was knocked down via Smad pathway. According to the different origins of the yolk sac as well as hemangiogenic lineages in mouse and human beings, the effect of TGF-β in these two species is not the same. Therefore, to examine the role of TGF-β in early human hematopoietic differentiation, it is necessary to use human embryonic stem cell-derived EB to recapitulate early stages of embryonic development [15]. To achieve this aim hematopoietic progenitors that were generated from EBs in transduced siTGF-βR2 and scramble groups were explored and compared with EBs in another group, with combination of defined cytokines. The authors attempted to determine whether TGF-β signaling inhibition could promote hematopoietic differentiation on early stage of differentiation in hEB-derived cells or not.

Material and Methods

Human ESCs Culture

The hESC line used in the present study, H6 (NIH code WA01) was obtained from Royan Research Institute. It is maintained on mitomycine inactivated mouse embryonic feeder cells in stem cells media. This media consists of DMEM/F12 (50:50, Invitrogen) supplemented with 20 % knockout serum replacement (SR, Gibco), 100 M nonessential amino acids (Gibco, 11140), 2 mM glutamine (Invitrogen, 35050), 50 U/mL penicillin, 50 g/mL streptomycin (Invitrogen, Grand Island, NY), 10^{-4} M β-mercaptoethanol (Sigma, St Louis, MO), and 20 ng/mL hbFGF (PeproTech, RockyHill, NJ, USA, 100-18B) in 25T cell culture flask Techno Plastic Products (TPP, 93100). When ESC colonies reached to 70–80 % confluency they were passaged enzymatically using a 1 mg/mL solution of collagenase type-IV (Gibco, 17104-019) for 3 min and the small clumps of ESC colonies were centrifuged at 1200 rpm for 5 min and transferred on to fresh feeder in a new flask.

hEB Generation

To generate EBs, H6 colonies were dissociated to small clusters ($\sim$10–20 cells) using collagenase IV (1 mg/mL) for 5 min followed by Trypsin–EDTA 0.05 % solution (Sigma, T4799) for approximately 2 min and were centrifuged at 1200 rpm for 5 min. The clusters were plated in 6-well non-treated-plate (JET BIOFIL®) in 2 mL aggregation media, consisting of Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Grand Island, NY) supplemented with 20 % fetal bovine serum (FBS), 100 M nonessential amino acids, 2 mM glutamine, 50 U/mL penicillin, 50 g/mL streptomycin, 10^{-4} M β-mercaptoethanol, 10 ng/mL BMP-4 (PeproTech, RockyHill, NJ, USA), and 50 g/mL ascorbic acid (Sigma). The aggregates were incubated at 37 °C in 5 % CO_2 atmosphere. Twenty-four hours later EB formation was checked by an invert microscope (Nikon, Eclipse TS100).

EB Harvest and Dedifferentiation

The next day after EB formation, same size EBs were harvested and replaced by the same medium supplemented with a cocktail of cytokines consisting of 100 ng/mL human stem cell factor (hSCF), 100 ng/mL human Flt3-ligand (hFlt3), 100 ng/mL of TPO, 10 ng/mL human Interleukin-3 (hIL-3), 10 ng/mL human Interleukin-6 (hIL-6), 50 ng/mL human granulocyte colony stimulating factor (hG-CSF), 10 ng/mL human bone morphogenetic protein-4

(hBMP4) (all of them from PeproTech, RockyHill, NJ, USA). The culture medium was changed every 3 days until day 15th for hEBs differentiation.

Human Umbilical Cord Blood (UCB) Purification

The UCB was transported to the laboratory by 150 mL transfer bags (Baxter, R4R2001). Then, 6 % Hetastarch in 0.9 % Sodium Chloride was added to the UCB at a concentration of 20 % blood volume in 50 mL centrifuge tube (TPP) and was kept for 2 h in laminar hood. The supernatant containing the desired nucleated cells was expressed off into a second falcon and after that, it was centrifuged at 1500 rpm for 5 min. The supernatant was added slowly to 3 mL Ficoll (Sigma-Aldrich®, Steinbach, Germany) and was centrifuged at 1800 rpm for 20 min in 25 °C. Then, UCB mononuclear cells (MNCs) from the interface were collected and washed twice in phosphate-buffered saline (PBS) and centrifuged at 1200 rpm for 5 min in 25 °C again. The supernatant was discarded and the MNCs were suspended in 5 mL RPMI containing 10 % FBS, then they were transferred to 25T flask. After 5 days, single cells were ready for the first passage [16].

Vector Construction and *Lentivirus* Production

Both piLenti-siTGF-βR2-GFP (i024469a) and Scrambled siRNA GFP lentiviral vector (LV015-G) were purchased from Applied Biosystems. After that, plasmids were propagated and purified using a QIAfilter Plasmid Maxi Kit (Qiagen, Germany).

Lentiviruses were produced by co-transfecting of HEK-293T cells with two plasmids separately viz. gag-pol expression plasmid, psPAX and VSV-G envelop plasmid. The pMDG.2 based on the calcium phosphate precipitation principle [12, 13]. Briefly, twenty-four hours prior to transfection, 4×10^6 HEK-293T cells were plated on 10 cm^2 plates [Techno Plastic Products (TPP), 93100] and incubated in humidified atmosphere at 37 °C, 5 % CO_2 overnight to reach 70–80 % confluence. The media was Dulbecco's Modified Eagle Medium (DMEM) a high glucose supplemented with 10 % FBS without antibiotics. Two hours prior to transfection, the medium was replaced with fresh medium without FBS and then co-transfected with 22.5 μg of eGFP-plasmid containing desired gene, 14.6 μg of psPAX, 7.9 μg of pMDG.2, 50 μL $CaCl_2$ 2.5 mM. Then, 450 μL of 2× HBS (140 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH = 7.05) was added and mixed gently and then incubated at room temperature (RT) for 20 min according to Trono's Lab instruction. The mixture was added drop-wise onto the HEK-293T cells. Before returning to the incubator, the plates were rocked back and forth and then sideways to achieve a uniform distribution of the precipitates throughout the plate. Sixteen hours later, the cells were washed once with DMEM and fresh medium containing 10 % FBS was added. The supernatant containing *Lentiviruses* was harvested 48–72 h post-transfection and was centrifuged to remove cell debris. Then it was filtered passing through 0.45 μm PVDF Durapore filter (Millipore, Bedford, MA). Viral supernatants were concentrated by ultracentrifugation at 50,000×*g* for 2 h in a SW-28 rotor (Beckman, Palo Alto, CA). The virus pellets were resuspended in the medium that was used to produce them. To estimate vector particles (p24) the enzyme-linked immunosorbent assay (ELISA) kit (DIA.PRO. Italy) was used. Virus titer was determined by fluorescence-activated cell sorting analysis of GFP positive HEK-293T cells and was approximately 2×10^9 transducing units (TU)/mL medium [17, 18].

Transduction of hESCs

At the day of transduction (day 0), H6 hESCs were washed with PBS and dissociated with 1 mg/mL collagenase type IV for 3 min and centrifuge at 1200 rpm for 5 min, then the supernatant was discarded. Dissociated cells and viral particles in mouse embryonic fibroblast-condition medium (MEF-CM) were transferred and incubated 2 h at 37 °C in CO_2 incubator, then all clumps were plated on mitomycin-C inactivated MEF feeder cells. Next day (day 1) medium was discarded and fresh viruses were added to H6 cells on MEF feeder with the same procedure. Six days after transduction GFP-positive colonies were picked up mechanically under fluorescence microscope (Nikon Multizoom AZ100 Multi-Purpose) for subsequent EB formation and molecular evaluation. ESCs were transduced with *Lentivirus* containing siTGF-βR2 and Scramble separately in two groups, as shown in Fig. 1.

Real-Time Polymerase Chain Reaction

Total RNA of EBs in all groups was extracted using BIOZOL Kit (BSC51M1) according to the manufacturer's instructions. The total extracted RNA was measured by NanoDrop ND-1000 (Peqlab, Erlangen, Germany). The cDNA was synthesized from 1 μg of total RNA using the PrimeScript™ RT reagent Kit (Takara Bio Inc, RR037A) at 37 °C for 15 min for the reverse transcription, 85 °C for 5 s to inactivate the reverse transcriptase (RT enzyme).

The sequence of oligonucleotide primers used in this study for gene amplification were obtained from previous published articles [7, 19–21] and some of them were designed using Perl Primer software (version 2.0.0.7). The sequences are shown in Table 1.

Relative transcript expression levels of all groups were measured by quantitative real-time PCR (Q-RT-PCR)

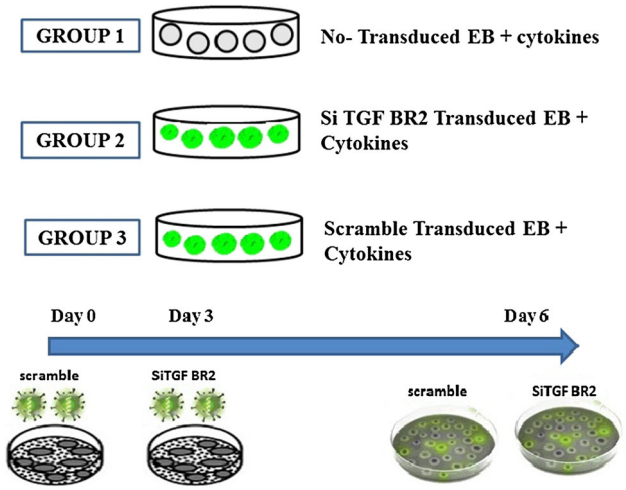

Fig. 1 The project plan of the study

using a SYBR Green master mix (Roche Applied Science, Indianapolis, IN, USA) based on method described by manufactures. The real-time PCR performed in 10 µL reaction contained: 5 µL of SYBR Green master mix, a 2.5 nM concentration of each forward and reverse primer, and 60 ng/µL of cDNA sample. Then, all micro-tubes were placed into Rotor-Gene 3000 (Corbett, Australia). The reaction protocol was as follows: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s (denaturation), 58–66 °C for 20 s (annealing), and 72 °C for 20 s (extension). Standard curves were generated and the mean threshold cycle (CT) values of each dilution in triplicate, and mean CT values of samples in duplicate were used for quantity calculation. The averages of fold change were calculated by differences in CT between samples and *GAPDH* gene (internal control). The relative quantification in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal control to normalize and a melting curve was acquired by heating the product to 95 °C, cooling to 55 °C and maintaining at 70 °C. The expression levels of hematopoietic progenitor specific transcripts (γ, ε-globin, and Brachyury), maturation markers (CD34, α, and β-globin) and stem cells specific transcripts POU5F1 (OCT4) were analyzed in the present study.

Statistical Analysis

The variance between groups was calculated by *T* test. The data were collected in Statistics programs for the Social Sciences software, (SPSS, Inc., Chicago, IL, USA) version 20. In the present study p value of ≤ 0.05 was considered statistically significant.

Table 1 The sequence of primers used for gene amplification

Primer names	Sequence	Product length (bp)	Accession number	Reference
OCT4-F	5′-ACATCGCCAATCAGCTTGG-3′	51	NM_001285987	Ratajczak et al. [19]
OCT4-R	5′-AGAACCATACTCGAACCACAT-3′			
TGF-βR2-F	5′-TTTTCCACCTGTGACAACCA-3′	342	NM_003242	Designed
TGF-βR2-R	5′-GCTGATGCCTGTCACTTGAA-3′			
Brachuary-F	5′-CCTCGAATCCACATAGTGAGAG-3′	117	NM_001270484	Designed
Brachuary-R	5′-AAGAGCTGTGATCTCCTCGT-3′			
CD34-F	5′-ACATCACAGAAACGACAGTC-3′	143	NM_001773	Designed
CD34-R	5′-TGTCTCTGGAGTTGAAACGT-3′			
GATA1-F	5′-CACTCCCCAGTCTTTCAG-3′	199	NM_002049	Designed
GATA1-R	5′-TCAAAGTCTCCAGGAAGC-3′			
ά-globin-F	5′-ACTCTTCTGGTCCCCACAG-3′	153	NM_000517	Ledran et al. [7]
ά-globin-R	5′-GTGGGGAAGGACAGGAAC-3′			
β-globin-F	5′-GCAACCTCAAACAGACACCA-3′	198	NM_000519	Ledran et al. [7]
β-globin-R	5′-TTAGGGTTGCCCATAACAGC-3′			
ε-globin-F	5′-ATATCTGCTTCCGACACAGC-3′	303	NM_005330	Ledran et al. [7]
ε-globin-R	5′-GCTTGAGGTTGTCCATGTTT-3′			
γ-globin-F	5′-CACAGAGGAGGACAAGGCTA-3′	238	NM_000184	Ledran et al. [7]
γ-globin-R	5′-CTTGAGATCATCCAGGTGCT-3′			
GAPDH-F	5′-CTCATTTCCTGGTATGACAACGA-3′	121	NM_001256799	Pakzad et al. [20]
GAPDH-R	5′-TTCCTCTTGTGCTCTTGCTG-3′			

Results and Discussion

Virus Production Carrying *siTGF-βR2* and Scramble

HEK-293T cells were transfected with a lentiviral plasmid expressing siTGF-βR2 and scramble separately by $CaPO_4$ precipitation method. In the present study 90 % efficiency in transfection were observed (Fig. 2A, B). To evaluate the optimal efficiency of *Lentiviruses* in transduction, concentrated lentiviral vectors at a multiplicity of infection (MOI) of 10 were used for transduction of human dermal fibroblasts (HDFs). After 72 h the results show high efficiency of eGFP expressing in transduced HDFs (Fig. 2C). The confirmed viruses were used for ESCs transduction according to the plane that is shown in Fig. 1.

EB Formation and Differentiation of HSC-Like Precursor In Vitro

Undifferentiated ESCs were infected with lentiviral expression vectors carrying siTGF-βR2 and its scramble separately. To follow the expression of siTGF-βR2 in vitro, the co-expression system based on co-translational separation of eGFP was used. Transduction was performed three times on each group with MOI 10. After 6 days, $eGFP^+$ colonies were scraped and dissected into small clusters under fluorescence microscope. As mentioned, in the present study during EB formation induction method was used for the differentiation of stem cells into the HSCs (Fig. 3). To promote hematopoiesis, the undifferentiated H6 cell clusters (transduced and non-transduced) were transferred to the EB media in three groups of siTGF-βR2, scrambled and non-transduced group. Twenty four h later, same size EBs from each group was transferred to differentiation media containing cytokines supportive for hematopoiesis. Following 15 days the EBs growth was checked by invert microscope in all groups. According to the authors' observations, the expression of siTGF-βR2 had no effect on EB size and viability as compared to non-transduced group. Figure 3 shows GFP^+ colonies and GFP^+ EB in these two transduced groups.

Knockdown of TGF-βR2 Can Promote Hematopoietic Differentiation

To determine the level of gene expression of siTGF-βR2, 6 days post-transduction ESCs in two siTGF-βR2 and scrambled groups were analyzed by real-time PCR and then infected ESCs cluster were taken to EB media. TGF-βR2 mRNA levels of EBs transduced with siTGF-βR2 *Lentivirus* showed significantly lower levels than the scrambled group, which can be a consequence of siRNA activity on gene silencing of TGF-βR2 (0.225313 ± 0.036646) vs. (0.913831 ± 0.007828) (Table 2). Figure 4 shows quantitative real-time PCR confirming the expression of TGF-βR2 to be reduced in 6 Day post transduction-ESC.

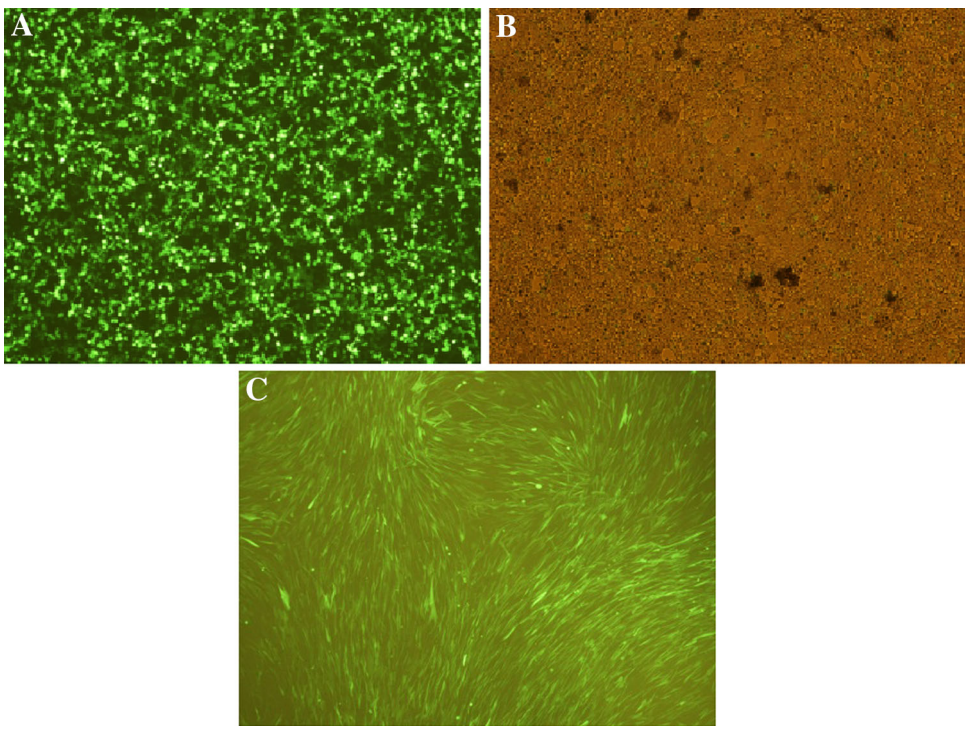

Fig. 2 **A**, **B** HEK-293T cells transfected with lentiviral plasmid expressing eGFP after 48 h, **C** HDFs transducted by eGFP *lentiviruses* after 72 h

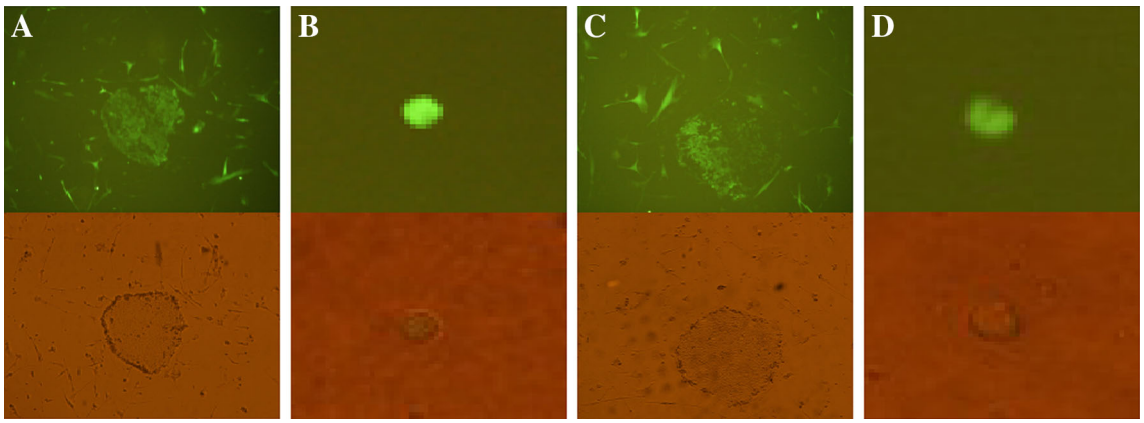

Fig. 3 **A** ESC colony transduced with scramble, **B** EB transduced with scramble in day 15, **C** ESC colony transduced with siTGF-βR2, **D** EB transduced with siTGF-βR2 in day 15

Table 2 CT values of GAPDH and TGF-βR2 detected by Q-RT-PCR

Sample	GAPDH CT value average	TGF-βR2 CT value average	$2^{-\Delta\Delta Ct}$ average
scr-siRNA	24.72 ± 0.2200	16.11 ± 0.1450	0.913831 ± 0.007828
TGF-βR2-siRNA	15.20 ± 0.2200	27.78 ± 0.5450	0.225313 ± 0.036646

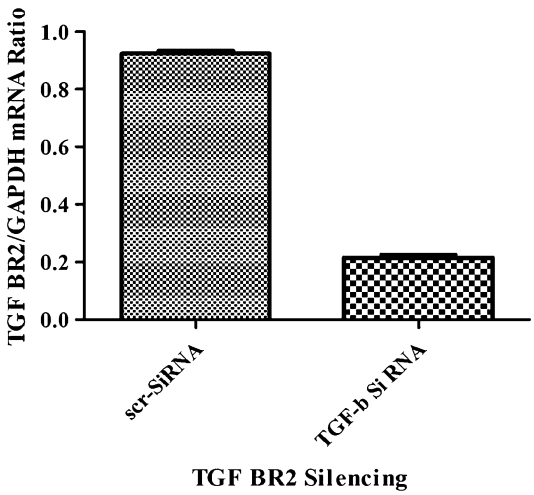

Fig. 4 The expression level of TGF-βR2 mRNA in EBs that transduced with siTGF-βR2 *Lentivirus* as compared to scramble group

Expression Pattern of Globin and Non-globin Markers of Hematopoietic Progenitor-Like Cells Expanded from EBs

To determine whether TGF signaling inhibition might promote hematopoietic progenitor development in EBs, the authors incubated EBs for 15 days and checked the expression level of hematopoietic specific genes in days 7 and 15 during EB differentiation. They examined known genes to be expressed at an early stage of hematopoietic differentiation using Q-RT-PCR. Core pluripotency factors such as POU5F1 (OCT4) were expectedly down regulated during EB formation and hematopoietic differentiation (15DEB: test vs. control -6.958 ± 0.05919 vs. -1.259 ± 0.08664; $p \leq 0.0310$) as shown in Fig. 5A. Furthermore, they found low expression of mesodermal marker like *Brachyury* gene in ESC but it was ascending in EB at 7 days and decreased during EB formation. The expression of *Brachyury* in siTGF group as compared with control group in EB at 7 days was significant (43.94 ± 1.414 vs. 19.82 ± 0.7072; $p \leq 0.0145$) but in EB at 15 day there was no significant difference as compared to the control group. To characterize potential hematopoietic cells initially, the authors focused on CD34 and GATA1 known as good markers of very early hematopoietic precursors derived from hESC [10]. The expression of *CD34* and *GATA1* genes were increased by partial inhibition of TGF signaling as compared to control group (4.7917 ± 0.1328 vs. 1.7715 ± 0.0061 respectively; $p \leq 0.032$ for CD34), (2.6019 ± 0.2069 vs. 0.45061 ± 0.4075 respectively; $p \leq 0.004$ for GATA1). Figure 5 shows the expression graph of these markers with the comparison to GAPDH. The expression pattern of four globins, epsilon and gamma (as fetal globins) and alpha and beta (as adult globins) was examined in cultured EB at days 7 and 15 in all groups as shown in Fig. 6. The present results determined that during the early stage of EB development, the expression level of epsilon and gamma globins was ascending which has been implicated in up-regulating embryonic globin synthesis in the human EBs. Alpha expression was detected slightly on the fifteenth day,

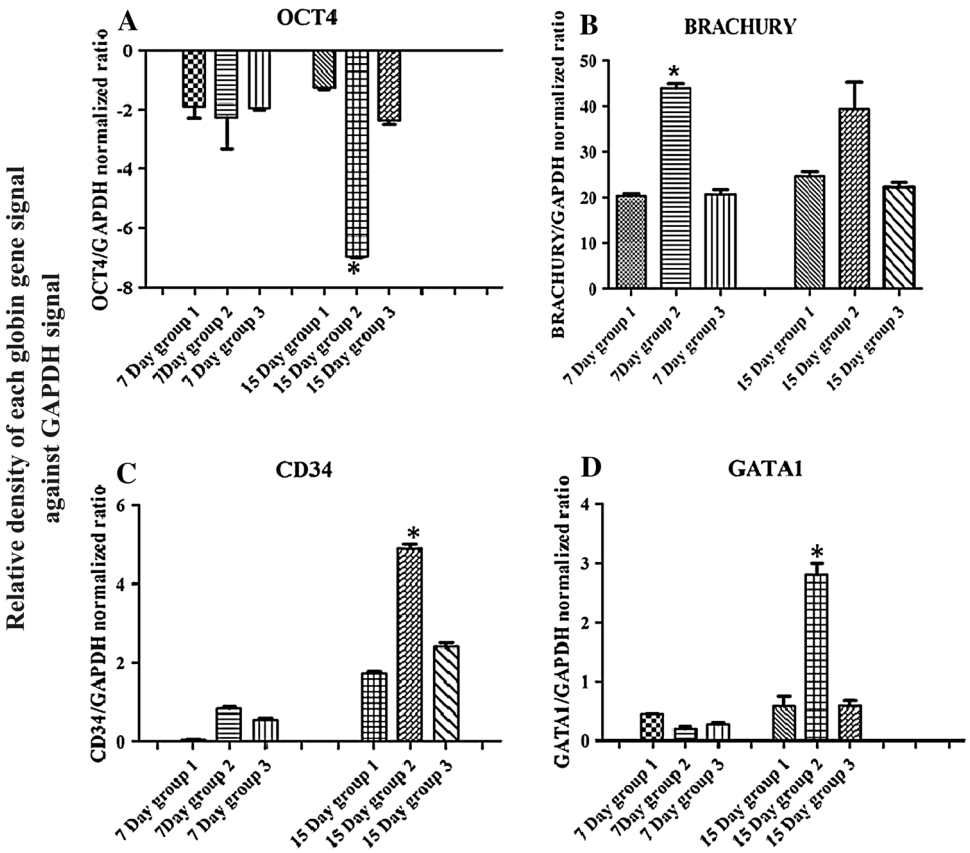

Fig. 5 Quantitative real-time PCR analysis of intracellular OCT4 (**A**), Brachyury (**B**), CD34 (**C**), and GATA1 (**D**) during EB differentiation reveals distinct changes in cells expressing siTGF-βR2 in EB cultured at seventh and fifteenth days. Expression is shown as mean ± SEM of three independent experiments and calibrate with the gene expression in undifferentiated human embryonic stem cells. Fold change in expression from undifferentiated hEB cells was calculated using the "$2^{-\Delta\Delta Ct}$ method" based on the CT for each target gene and internal normalizations with GAPDH. N = 3, significance between each sample and the control (group 1) was calculated by Student's *t* test

but the expression of β-globin mRNA was not significantly altered as compared with HSC.

In the present work, the authors have attempted to clarify the influence of TGF-β signaling inhibition in the early hematopoietic differentiation potential of hEB-derived cells. For this approach lentiviral expression of siTGF-βR2 was used. The differentiation technique included EB formation in combination with cytokines supportive for hematopoiesis. The selective marker to distinguish transduction efficiency was the co-expression of GFP by siTGF-βR2. At least 7 and 15 days EB were characterized following differentiation by focusing on some hematopoietic marker expression such as *CD34*, *GATA1* and *globins* by real-time PCR. The significant outcome of this project was the influence of partial TGF-β signaling inhibition to promote hematopoietic differentiation.

In the past two decades, scientists have been attempting to provide a therapeutic alternative to bone marrow transplants for high-risk leukemia and a range of genetic blood disorders [22]. HSCs derived from human embryonic stem cells (hESCs) could be appropriate alternative in this goal. HSCs could give rise to erythroid, myeloid, and lymphoid lineages but the efficiency of available protocols is low. For producing the functional HSCs that can reconstitute all hematopoietic lineages in vivo, it is necessary to understand the regulatory network of genes which interfere on the differentiation pathway of HSCs and attempt to optimize procedure for differentiation [23].

TGF-β and associated members of the family have diverse effects on hematopoietic differentiation. These isoforms (TGF-β1, 2, and 3) have distinct but overlapping effects on hematopoiesis. Depending on the differentiation stage, TGF can act as inhibitor or accelerator. Previous studies have shown the inhibitory effect of TGF-β on early stage of hematopoietic differentiation and HSC proliferation.

Many studies have been done to investigate the role of TGF-β1 (the isotope of TGF-β) in early development of hemangioblast. These cells are thought to be a bipotent mesodermal ancestor of endothelial and hematopoietic cells [24]. TGF-β super-family regulates both stemness and various cell differentiation pathways among hemangioblast. The same molecular profiles and temporal developmental patterns which have been detected in endothelial/hematopoietic precursors derived from hEBs [6, 7, 25]. Poon et al. have examined the effect of TGF-β1 on differentiation of endothelial precursors from ESCs. They evaluated EB treatment with TGF-β1 on early

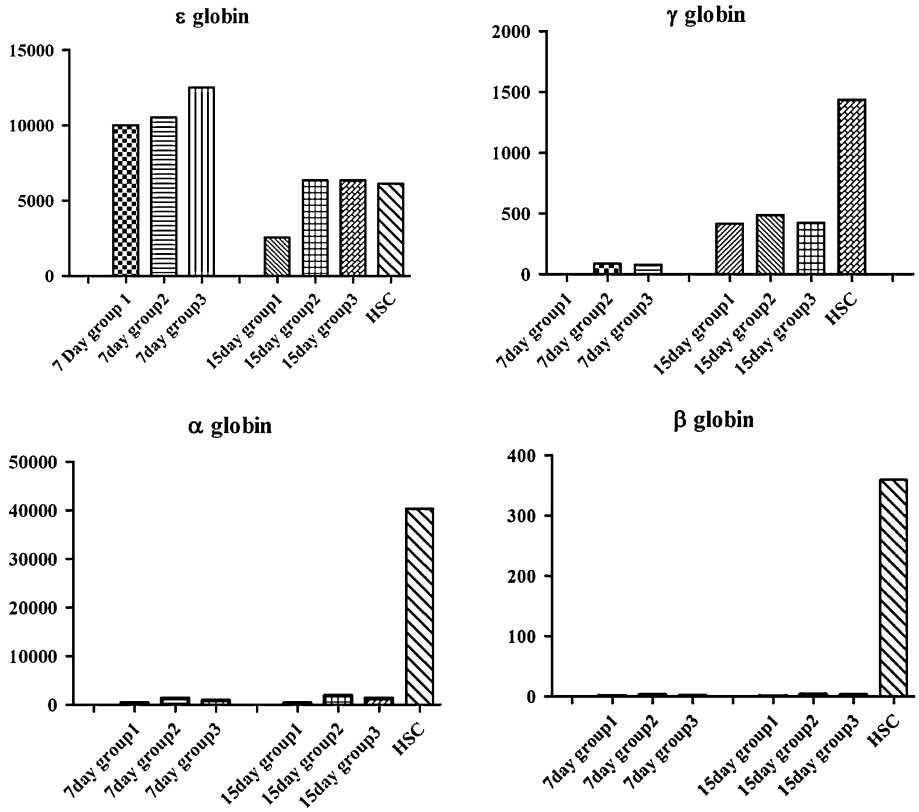

Fig. 6 Quantitative real-time PCR analysis of embryonic (ε), fetal (γ), and adult (β and α) globin expression during EB differentiation. The relative level of each globin mRNA is shown as fold value of the level of each globin mRNA in ESCs. Results were performed in triplicate and mRNA levels were normalized to GAPDH

differentiation showing that TGF-β1 can deplete the expression of endothelial precursor marker [12, 15]. In addition TGF-β1 treatment of day 13 EBs are assessed for CD34 and GATA2 RNA levels, the two hematopoietic markers which can be expressed at low levels during early EB development [25]. Interestingly, EB treated with TGF-β at early differentiation stage, prevents expression of endothelial and hematopoietic markers [15].

In the adult blood system, TGF-β1 is known to regulate G1/S cell-cycle progression to maintain a primitive and undifferentiated population of HSC progenitors [26]. Additionally, TGF-β1 inhibits the in vitro HSC proliferation. Delayed addition of TGF-β1 until the HSC had undergone three divisions (eight cells/well) was still able to cause growth arrest of the cells at that stage. Examining the effects of TGF-β on hematopoietic cell growth in long-term bone marrow cultures (LTBMCs) has shown the production of TGF-β by stromal cells which has a negative-feedback loop to maintain stem cell quiescence and to inhibit progenitor cell cycling [27]. Addition of neutralizing TGF-β antibodies to hematopoietic cell growth in LTBMCs resulted in increased output of mature myeloid cells. The neutralization of TGF-β in hematopoietic stem/progenitor cells derived from UCB cells in vitro has given the therapeutic potential to these cells [28]. The same subsequent experiments have shown that morpholino antisense nucleotides (MAS) to TGF-β1 or TGF-βRII induce HSC survival, reduce the number of HSC if required for hematopoietic repopulation, and decrease the time for engraftment [27]. A direct inhibitory effect of TGF-β1 on purified CD34 human stem/progenitor cells has also been reported [29]. Depending on the cell type in the late differentiation TGF-β accelerated the dendritic cell differentiation from common dendritic cell progenitors [30]. However, TGF-β repressed development of natural killer (NK) cells from CD34(+) progenitors, and inhibited differentiation of CD16(+) NK cells [31].

Conclusions

According to the information obtained from previous researches about negative regulatory performance of TGF-β during differentiation, it has been mentioned that TGF-βR2 transcripts could be detected in undifferentiated human ESCs [12, 15]. In the present study, the TGF regulatory activities were reduced during differentiation through knockdown TGF-βR2 expression to promote hemangioblast formation. Real-time PCR analysis on the expression level of hematopoietic-specific genes has shown 2–3-folds increase in EBs after TGF signaling inhibition. The findings of the present study may confirm the

assumption that TGF receptor silencing can affect the inhibitory role of TGF-β during hematopoietic differentiation. Many questions about the features and the roles of Smad pathway co-factors in ESCs stemness or differentiation have not completely answered yet [12]. On the other hand, the transcriptional cascade(s) switched on/off by Smad-specific signaling pathways is/are still missing in human ESCs. Therefore, to ensure the present hypothesis, it is necessary to examine the effects of other sub-family of TGF-β like Smad2 or Smad7 in Smad pathway.

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